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14. ABSTRACT

To date, most cancer research has focused on alterations in the sequence, gene structure, copy number and expression of protein coding genes. However, we find increasingly that the genome generates a diversity of non-coding RNAs, many of which have unknown functions. MicroRNAs (miRNAs), which are small, 21-24nt RNAs generated by the key enzyme Dicer, represent a prominent class of such non-coding RNAs. There's evidence suggesting that miRNAs could collaborate with oncogenes to facilitate tumor formation. On the other hand, several miRNAs are found to function as tumor suppressors. Our previous study revealed a miRNA family, miR-34, as direct transcriptional target of p53, the master tumor suppressor gene. To address the role of miR-34in cancer formation and maintenance, we generated cell lines over express miR-34. We have demonstrated that ectopic expression of miR-34 in both primary and tumor cell lines can induce growth arrest through repression of cell cycle genes, and we have shown in animal model that tumor cells over expressing miR-34 have disadvantage in tumor initiation and maintenance. Our work placed miRNAs as one of the central mediators of p53 tumor suppressor network, which plays an important role in many cancer types, including breast cancer.

15. SUBJECT TERMS
Dicer, microRNA, mir-34, mammary tumor stem cell

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Introduction

Cancer arises from genetic lesions that result in uncontrolled proliferation, cell survival, loss of differentiation and invasive growth. So far, cancer studies have focused on genetic alterations in protein coding genes. It is only recently that non-coding RNAs, in particular, microRNAs (miRNAs) have been shown to play important roles in cancer. Since then, a number of studies further support the idea that miRNAs can be components of oncogenic and tumor suppressor networks.

MiRNAs are small, non-coding RNAs which regulate gene expression through post-transcriptional repression. Nascent miRNA transcripts (pri-miRNAs) are first transcribed from the genome, and then processed sequentially by two key ribonuclease III enzymes, Drosha and Dicer, to generate mature miRNAs duplexes which are 21nt to 24nt in length. Usually, one strand from the miRNA duplex is incorporated into the effector complex, the RNA-induced silencing complex (RISC). RISC recognizes specific target mRNAs through imperfect base-pairing, and down-regulates their expression by post-transcriptional gene silencing.

MiRNAs recognize their target genes by binding to their complementary base-pairing sites on the target mRNA. A series of mutational analyses indicated that the most critical interactions between the miRNA and its targets occur within the 5' region of the miRNA. Therefore, the eight nucleotides at the 5' end of a miRNA are designated as the "seed" sequence, whose complementarity to the target mRNA has been employed to search for candidate targets. In a recent study by Lewis et al., more than 5300 human genes are predicted as conserved miRNA targets, representing 30% of human genome.

Increasing evidence has suggested that miRNAs are components of oncogene and tumor suppressor pathways. Inappropriate expression and structural alterations of miRNA genes have been found in a variety of tumor types, and several functional studies have shown the oncogenic or tumor-suppressive potential of specific miRNA families. Our study uncovered the miR-34 family of miRNAs in the p53 tumor suppressor network. The functional study of miR-34 has shown that miR-34 possess anti-proliferative potential by repressing cell cycle genes. Deletion of miRNAs of the miR-34 family has been reported in several human tumours and cancer cell lines. And our animal data has indicated miR-34 may act as tumor suppressor which may afford new opportunities for diagnosis and treatment of human cancer.

Body

Creation of Dicer deficient cells and animals

Dicer is a ribonuclease enzyme that is involved in the biogenesis of miRNAs and other small RNAs. To elucidate the role of Dicer and miRNAs in mammals, and particularly in cancer, we created embryonic stem (ES) cells with a conditional targeted allele of Dicer. We found that once induced to delete Dicer, these cells were deficient in proliferation. Indeed, most Dicer deficient ES cell clones were unable to outgrow.

To further investigate this phenotype, we used the ES cells to establish a mouse line harboring the conditional Dicer allele. This system was used to delete Dicer in various adult and developmental compartments, including oocytes, skin and brain. Interestingly, we found that there is a requirement for Dicer for the correct assembly and function of the meiotic spindle in ooyctes.

Over-expression of *mir-34* induces cell cycle arrest

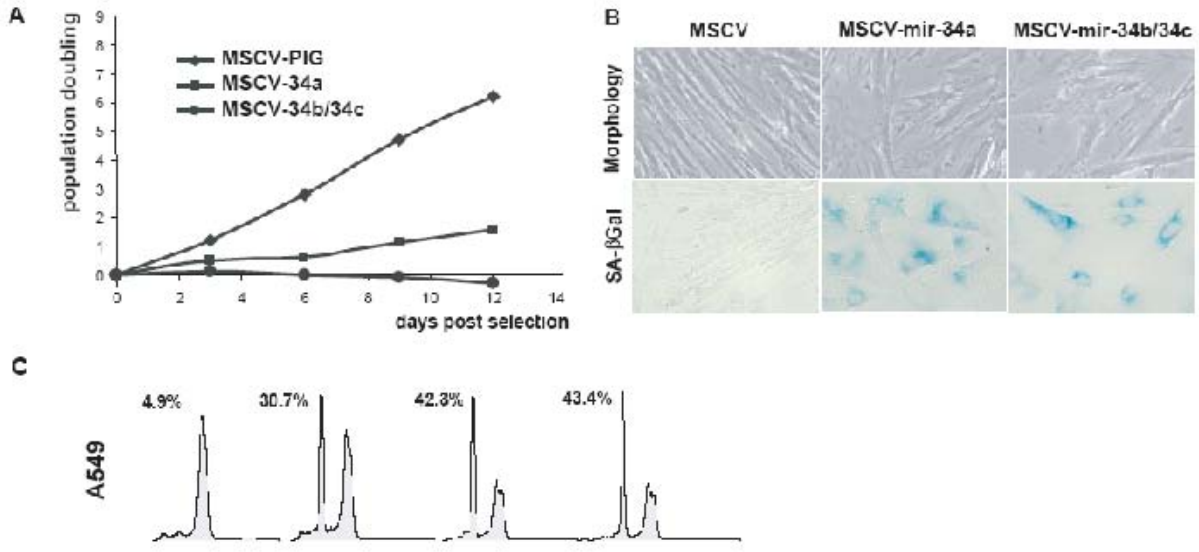


Fig1. miR-34 family miRNAs mediate growth arrest in primary and tumor cell lines. A. Proliferation of human fibroblast cells (IMR90) was measured as cumulative population doublings following retroviral delivery of vectors directing expression of primary *mir-34a, mir-34b/c* or a control. **B.** IMR90 cells engineered to express pri-*mir-34a* or pri-*mir-34b/c* exhibited morphological alterations similar to those seen in senescent cells and stained with SA-β-gal **C.** A549 were transfected with *mir-34*-mimetic siRNAs and cell cycle arrest was measured using a G2 trapping assay. Briefly, cells were treated with Nocodazole 24 hours post transfection. PI staining and FACS analysis were performed to monitor cell cycle distribution 16-20 hours after Nocodazole treatment. Proliferating cells accumulate in G2 phase, and only cells arrested in G1 or S prior to Nocodazole treatment retain those states.

In our previous study, we identified a miRNA family, miR-34 a, b, c as direct transcriptional target of p53. To further investigate the function of miR-34, we generated human fibroblast cell

lines expressing ectopic miR-34. The two major end effects of p53 activation are apoptosis and growth arrest, either transient (cell cycle arrest) or permanent (senescence). We found that, consistent with their function as p53 downstream effectors, ectopic expression of either miR-34a or miR-34b/c led to a substantial growth inhibition (Fig. 1A). And we noted distinctive morphological alterations characteristic of cellular senescence in the cells (Fig. 1B, top panel). In accord with this observation, about 60% of the cells were positive (blue) for a senescence marker, SA-β-gal, at 6 days post selection ((Fig. 1B bottom panel). Similar effects were noted in mouse fibroblast cells (data not shown). Ectopic expression of miR-34 genes also led to growth arrest in a panel of tumor cell lines including HCT116 and A549 (Fig 1C).

<u>miR-34 down regulates cell cycle genes</u>

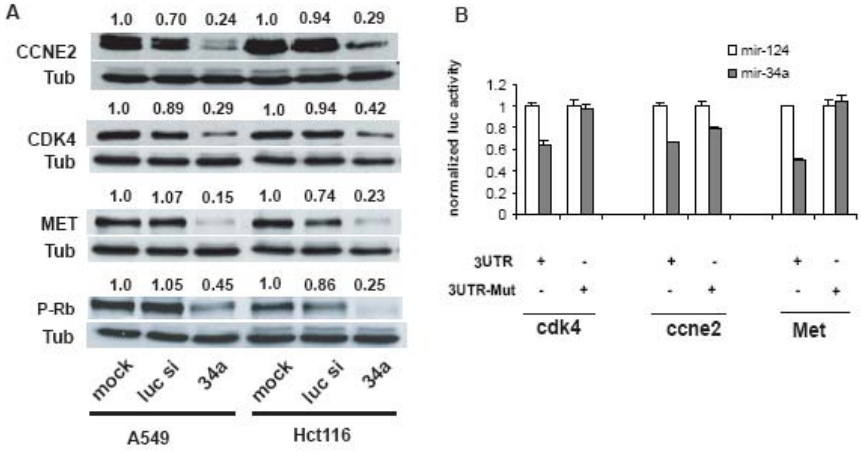
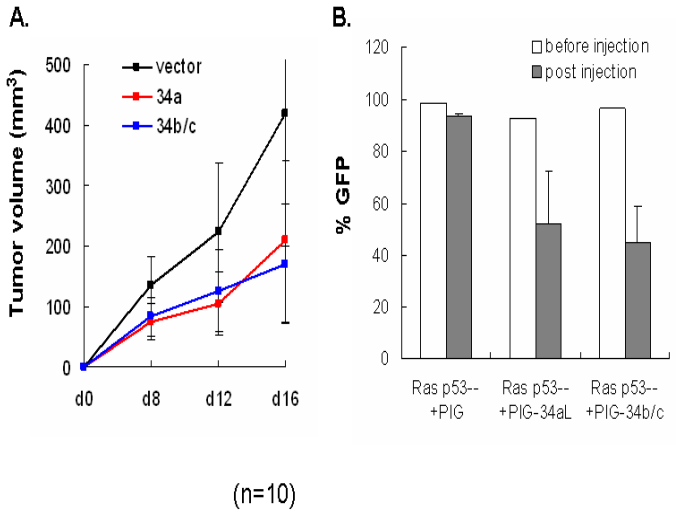


Fig2. *mir-34* regulates some cell cycle gene. A. Western blots measuring protein levels following *mir-34* delivery for multiple candidate targets identified in the cell cycle overlap gene set. Protein lysate were collected after 48 hrs of siRNAs transient transfection. As a consequence of cdk and cyclins down-regulation, Rb phosphoralation is also reduced. **B.** luciferase reporter assay confirms the *mir-34* targets. Wild type and seed region mutated 3-UTR fusing to a *firefly* luciferase reporter were co-transfected with *mir-34* or *mir-124* into Hela cells, *Renilla* luciferase construct was cotransfected as internal control. Cells were lysed and luciferase activities were measured 24 hrs post transfection.

To determine the mechanisms through which miR-34 family contributes to growth arrest, a list of candidate targets of miR-34 was collected by bioinformatics approaches. Next, we used Western Blots to examine the protein levels for selected candidate miR-34 targets in two tumor cell lines: HCT116 and A549, transfected with *mir-34* miRNA. To exclude the possibility that protein level down-regulation is due to non-specific transfection effects, GL3 siRNA against luciferase and miR-124 miRNA (which should have no effects on miR-34 targets) were used as controls for transfection. For those miR-34 targets, we expect that their protein levels will be specifically suppressed in miR-34 transfected cells. In our experiment, we found that a selection of candidate miR-34 targets, including cyclin E2 (Ccne2), cdk4, Met, revealed specific inhibition (Fig 2A). To test whether the regulation is direct, luciferase reporter assays were performed. The 3'-UTRs of cyclinE2, cdk4 and Met were cloned from genomic DNA, and their miR-34 seed complimentary sites were mutated. The level of luciferase of the three reporters were all reduced specifically upon miR-34 transfection, while the repression was eliminated when we mutated the miR-34 seed compliments in the cdk4 and met 3'-UTRs (Fig 2B)

Over expression of miR-34 has disadvantage in tumorigenesis



(n=10)

Fig3. over-expressing mir-34 has disadvantage in tumor progression (A) Over-expressing mir34a or mir34b/c clusters resulted in delayed tumor growth. P53-/-; Ras liver progenitor cells were infected with mir34 harboring retrovirus (PIG vector) and selected with 2.5ug/ml puromycin for 2 days. $3X10^6$ cells were injected in nude mice and tumor volume was measured by caliper. Value are mean+-s.d. (n=10). (B) Negative selection against mir34 in the tumors. The percentage of cells retaining mir-34 retroviruses were quantified by GFP expressed from the PIG vector. D16 tumors (post injection) were digested by dispase to generate single cell suspension and sorted by FACS. The decrease of %GFP post injection indicated that either some tumor cells have lost mir-34 expression or the non-infected cells escaping the puro selection have taken over the tumor population.

The observation that miR-34 induces grwoth arrest by repressing cell cycle genes trigered us to analyze whether miR-34 could act as a tumor suppressor *in vivo*. Thus, we transduced the *p53* null mouse hepatoblasts by retrovirus expressing control or miR-34s, together with oncogenic *ras* (*HrasV12*). And the cells form tumors when grafted into a permissive environment in a recipient mouse. Tumor growth was measured by size and the percentage of GFP which represents miR-34 expression. As in Fig 3A, both miR-34a and miR-34b/c overexpression significantly suppressed tumor growth. Although tumors in the miR-34 group eventually grow to a significant size, we saw a decrease of GFP, in the tumors at day 16 compared to day 0 (Fig 3B), indicating the tumors select against miR-34 expressing cells.

Next, to address the role of miR-34 in tumor maintenance, we generated mouse liver tumor cells harboring tet-on miR-34. The miRNA is not expressed in the absence of tetracycline (or its analog, Doxycycline, Dox) but is acute induced upon Dox treatment. Upon tumor manifestation, animals will be treated with Dox or left untreated, and tumor growth will be monitored by bioluminescence imaging through luciferase. It was soon apparent that tumors regressed after miR-34 activation (Fig 4). Overall, these results imply that miR-34 may act as tumor suppressors *in vivo*.

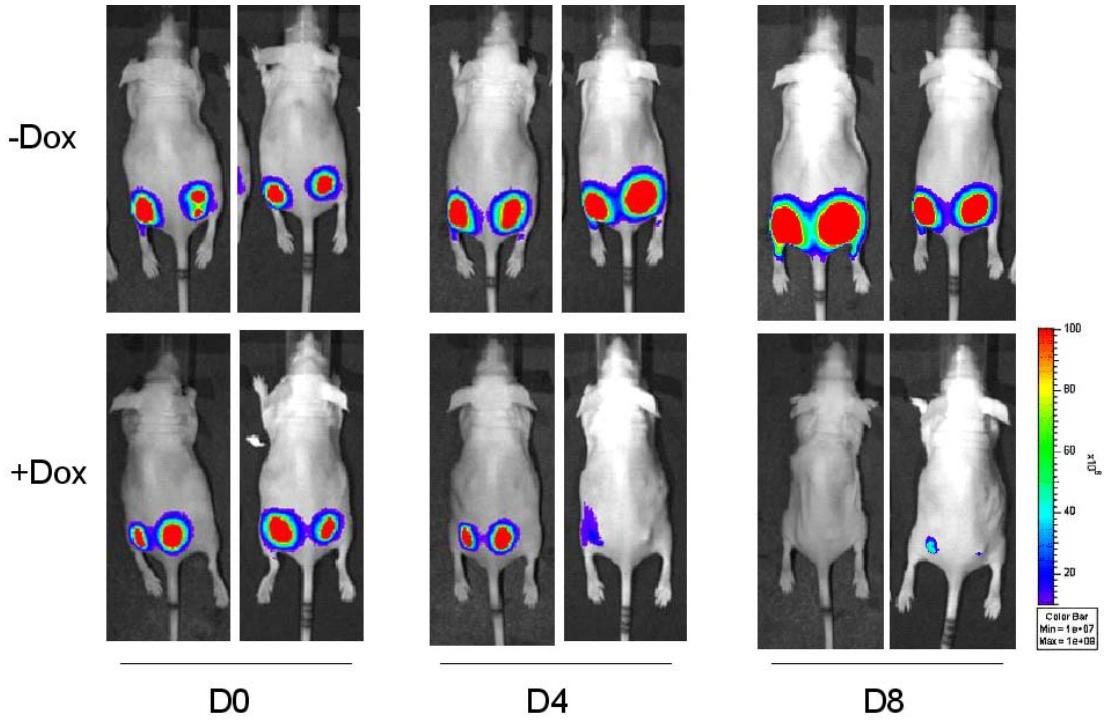


Fig4. Acute induction of mir34-a results in tumor regression.
p53 null and Ras expressing murine liver tumor cells are infected with tet-on mir-34a retrovirus and injected into nude mice. The animals are either untreated (-Dox, upper panel) or treated with Doxycyclin (+Dox, lower panel) to induce the expression of mir-34a *in vivo.* Bioluminescence pictures of two representative animals in each group are shown.

Key Research Accomplishments

- The generation of Dicer deficient cell lines and mice
- The identification of a miRNA family, miR-34, as direct transcriptional target of p53 tumor suppressor
- Ectopic expression of miR-34 induces growth arrest in both primary and tumor cell lines
- MiR-34 regulate cell cycle genes through post transcriptional repression
- Tumor cells over expressing miR-34 have disadvantage in tumor initiation and maintenance

Reportable outcomes

Manuscripts

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Conclusion

Dicer is a key enzyme in the biogenesis of miRNAs and other small RNAs. We generated genetic tools for studying the role of Dicer in mammals. In particular, we found that Dicer is required for the survival and proliferation of embryonic stem cells, and for spindle integrity in mouse oocytes. Dicer deficient cells and mice are an important resource for the community, and provide an interesting opportunity to study the global role of miRNAs in cancer.

P53 responds to DNA damage or deregulation of mitogenic oncogenes through the induction of cell cycle checkpoints, apoptosis, or cellular senescence. Mutations in *p53* are often associated with aggressive tumor behavior and poor patient prognosis. The p53 tumor suppressor network has been intensively studied; however, genetic analyses long hinted at the existence of components that remained elusive. For example, although p53 is clearly a transcriptional activator, numerous reports indicated that p53 also represses the expression of specific genes either directly or indirectly. The manner in which this occurred was obscure, with both transcriptional and posttranscriptional suppression as possible mechanisms. In the latter case, the discovery of extensive networks of miRNAs, offered the possibility that p53-mediated control of miRNA expression could allow it to act indirectly to repress target gene expression at the posttranscriptional level.

Our studies have identified miR-34 as a miRNA component of the p53 network, for the first time revealing interplay between proteins and non-coding RNAs in this pivotal tumor-suppressor pathway. Ectopic expression of miR-34 recapitulates the biological effects of p53, including growth arrest in our study and apoptosis by several recent studies, through its ability to dampen the expression of pro-proliferation and anti-apoptotic genes. Along with the report that deletion of miR-34 family miRNAs has been found in many cancer types, our animal work implies miR-34 as potential tumor suppressor. These findings suggest that miRNAs, and in a broader sense non-coding RNAs, may be previously unrecognized but integral components of established oncogene and tumor-suppressor networks.

Thus, it is critical to explore miRNA's value as novel therapeutical targets and/or diagnosis markers. Since the *in vivo* delivery of sequence-specific miRNA mimics and antagonists has been gaining great appreciation, it's technically possible to express or inhibit certain miRNAs in tumors and test their role in tumor maintenance. In addition, large-scale expression studies of miRNA profiles in multiple human tumor types have revealed that miRNA signatures are correlated with the developmental lineage and differentiation status of various tumors. Moreover, miRNA signatures can be used to identify certain poorly differentiated tumors, many of which were difficult to be classified based on mRNA profiles. Such findings suggest an unexpected potential of miRNAs as diagnostic tools, and possibly therapeutic targets.

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